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An "off-the shelf" Synthetic Membrane to Simplify Regeneration of Damaged Corneas

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***Abstract*— Our overall aim is to develop a synthetic off-the-shelf alternative to human amniotic membrane which is currently used for delivering cultured limbal stem cells to the cornea in patients who suffer scarring of the cornea because of the loss of limbal stem cells. We have recently reported that both cultured cells and limbal explants grow well on electrospun Poly(D,L-lactide-co-glycolide) (PLGA) (44 kg/mol) with a 50:50 ratio of lactide and glycolide and sterilized with γ-irradiation. Prior to undertaking a clinical study our immediate aim now is to achieve long term storage of the membranes in convenient to use packaging. Membranes were electrospun from Poly(D,L-lactide-co-glycolide) (44 kg/mol) with a 50:50 ratio of lactide and glycolide and sterilized with γ-irradiation and then stored dry (with desiccant) for several months at -80°C and -20°C , Room temperature (UK and India), 37°C and 50°C. We explored the contribution of vacuum sealing and the use of a medical grade bag (PET/Foil/LDPE) to achieve a longer shelf life. Confirmation of membranes being suitable for clinical use was obtained by culturing tissue explants on membranes post storage. When scaffolds were stored dry the rate of breakdown was both temperature and time dependent. At -20°C and -80°C there was no change in fiber diameter over 18 months of storage, and membranes were stable for 12 months at 4°C while at 50°C (above the transition temperature for PLGA) scaffolds lost integrity after several weeks. The use of vacuum packaging and a medical grade bag both improved the storage shelf-life of the scaffolds. The impact of temperature on storage is summarized beneath. We report that this synthetic membrane can be used as an off-the-shelf or-out-of-the freezer alternative to the amniotic membrane for corneal regeneration.**

I. Introduction

Limbal stem cell loss can be caused by a number of conditions such as trauma, thermal and chemical burns, and autoimmune disorders [1] and results in scar tissue covering the cornea with reduced or even complete loss of vision [1-3]. The front line treatment is transplantation of a monolayer of limbal cells on a human amniotic membrane obtained from tissue banks. Culture of cells from the contralateral unaffected eye is undertaken wherever possible or from donor eyes (with associated immunosuppression) when no autologous cells are available [3-4]. This membrane degrades over several weeks leaving these cells in place. However, the use of the amniotic membrane is associated with some limitations such as the need to establish a donor tissue bank, the risk of disease transmission, and variation in donor tissue and processing [5-7].

Consequently, the aim of this research is to make a synthetic biodegradable alternative membrane, available as a readily stored product, to replace the use of human amniotic membrane to reduce risks of disease transmission and to improve the accessibility and reproducibility of these membranes for surgeons.

In this study poly(D,L-lactide-co-glycolide) (PLGA) was selected as it is biodegradable and biocompatible, FDA approved and has been used for many years in products such as dissolvable sutures [8]. While there are numerous publications on the development of biodegradable electrospun membrane for clinical use there are still very few that have addressed the issues of how to store these membranes. This study pays special attention to the production and storage of biodegradable PLGA membranes.

With respect to sterilization of membranes, γ-irradiation is the most widely accepted sterilization methodology [9-13]. All sterilization methodologies are likely to affect the physical properties of electrospun membranes; hence it is important to assess the effect of γ-irradiation on the storage of the membranes. Accordingly we looked at the stability of membranes when stored dry at a range of temperatures, again looking to what extent γ-irradiation affected stability on storage for durations of up to 18 months. Additionally, a 12 months study was carried out to explore the contribution of vacuum sealing and the use of a medical grade bag (PET/Foil/LDPE) to achieve a longer shelf life. Thus in summary this study provides basic information on the storage of membranes which are being developed to be an off-the-shelf replacement for the amniotic membrane.

II. Materials and Methods

A. Polymer and electrospinning

For this study Poly (D,L-lactide-co-glycolide) with a 50:50 ratio of PLA to PGA (Purac; PLG5004 (M_w 44 kg/mol) was electrospun. These PLGA membranes were electrospun to our specifications by The Electrospinning Company (Didcot, Oxford), with fiber diameters of 2-3 μm

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and a scaffold depth of 50 µm. 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Sigma Aldrich , Dorset, UK) was used as a solvent and polymers were dissolved at room temperature to produce solutions of suitable viscosity for electrospinning (10 wt%).

A programmable Harvard PHD4400 syringe pump (Harvard Apparatus, Kent, UK) was used to deliver polymer solutions from a 5 ml plastic syringe (Becton Dickinson, Oxford, UK). Polymers were delivered at a constant rate of 800 µl/h, via PTFE tubing (1/16" O.D.), to a blunt tipped stainless steel needle with an internal diameter of 0.8 mm (Intertronics, Kidlington, Oxfordshire, UK). The tip of the needle was connected to a positive high voltage unit (Glassman High Voltage Inc. High Bridge, NJ, USA) and solutions were electrospun with an applied voltage of 12.5 kV. Fibers were deposited onto a grounded, custom built rotating mandrel (120 mm in diameter, 250 mm in length) at a distance of 300 mm from the tip of the needle, coated in 50 µm thick aluminium foil.

Electrospinning was performed in an environmentally controlled, A1-Safetech, air recirculation cabinet (a1-envirosciences GmbH, Düsseldorf, Germany) at an air temperature of 25˚C and a relative humidity of 25%. In order to make sure no residual solvent remained in the membrane a vacuum oven was used to dry membranes at room temperature for 48 hours. Membranes were then cut into 22 mm diameter discs and placed in 12-well, non-tissue culture treated plates (BD Falcon, Beckton Dickinson, Oxford, UK). The plates were then individually sealed in polypropylene bags (Andrew James UK LTD, Bowburn, UK) before storage.

B. γ-irradiation of membranes

γ-irradiation was used to sterilize PLGA membranes which were previously fabricated and mounted in 12-well plates by The Electrospinning Company. This process was carried out at Synergy Health Plc. (Moray Road, Swindon, UK), with an external dose range of 25-40 kGy. The PLGA membranes (sterilised and non-sterilised) were then compared throughout to assess the impact of sterilization on their storage when stored without cells at a range of temperatures.

C. Storage of membranes

γ-irradiated and non-sterile PLGA membranes were stored at a wide range of temperatures from -80°C to +50°C in order to assess the storage shelf life of the membranes over 18 months using SEM to assess fiber integrity. Additional membranes were stored at 37°C (both in a dry oven and a humidified incubator). In the case of the latter, membranes were placed in an empty petri dish within an incubator containing 5% CO_2 with high humidity.

A similar storage experiment was carried out at the same range of temperatures for 12 months and membranes were vacuumed within polypropylene (PE/PA composite) vacuum sealed bags (Andrew James UK LTD, Bowburn, UK) with thickness of 0.12mm. Water absorption by membranes was detected using silica orange, cobalt (II) chloride and copper (II) sulphate desiccants. Finally, another set of storage experiment using vacuum packaging and a high moisture barrier medical grade bag (PET/Foil/LDPE) (Riverside Medical Ltd. Derby, UK) were undertaken to improve the storage shelf-life of membranes.

D. Data acquisition by SEM

Scanning electron microscope (Philips/FEI XL-20 SEM) was used in this study to examine the morphology, microstructure, fiber diameter and fiber integrity of PLGA membranes (non-sterile and γ-irradiated) at an accelerating voltage of 10–15 kV. Samples were mounted on 12.5 mm stubs. Finally the samples were sputter coated with approximately 25 nm of gold and then examined using SEM. Image J software was used in order to measure the fiber diameter of PLGA membranes.

E. Statistical analysis

Results were tested for normality using a Kolmogorov Smirnov test. Results that showed normal distribution ($p > 0.05$) were analysed using SPSS via a Oneway Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed results ($p < 0.05$). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

III. RESULTS AND DISCUSSION

PLGA membranes were sterilized with γ-irradiation and then stored dry (with desiccant) for 18 months at -80°C, -20°C, 4°C, room temperature, 37°C and 50°C. We explored the contribution of vacuum sealing and the use of a medical grade bag (PET/Foil/LDPE) to achieve a longer shelf life.

A. Storage of membranes - γ-irradiated vs. non-irradiated

On the basis of SEM images, fiber integrity was assessed as fully intact (+++), some indication of fiber swelling (++), fibers merging (+) or no evidence of intact fibers remaining (-). Table 1 summarizes all storage experiments for these biodegradable membranes. At 50°C membranes had completely collapsed by 1 week whether sterilised or not. At 37°C (under dry conditions) membranes showed some evidence of breakdown by 2 weeks and under moist conditions fibers had completely merged by 2 weeks. Placing membranes within a moist environment at 37°C resulted in a rapid collapse of the fibers, whereas the same membranes maintained in culture media (with a bicarbonate pH buffering

Table 1. Fiber integrity of non-sterile and γ-irradiated electrospun PLGA (50/50) membranes (M_w:44 kg/mol) at various temperatures during storage over periods of 18 months. Membrane integrity scored as fully intact fiber (+++), some fiber swelling (++), fiber merging (+) or no intact fiber (-).

PLGA Scaffolds	Temperature (°C)	Fiber Integrity: Months 0	1	3	6	9	12	18
Non-Sterile (44 kg/mol)	50°C	+++	-	-	-	-	-	-
	37°C (Dry)	+++	+	-	-	-	-	-
	37°C (Moist)	+++	-	-	-	-	-	-
	21°C (Room)	+++	+++	+++	++	++	++	++
	4°C	+++	+++	+++	+++	++	++	++
	-20°C	+++	+++	+++	+++	+++	+++	+++
	-80°C	+++	+++	+++	+++	+++	+++	+++
γ-irradiated (44 kg/mol)	50°C	+++	-	-	-	-	-	–
	37°C (Dry)	+++	+	-	-	-	-	–
	37°C (Moist)	+++	-	-	-	-	-	–
	21°C (Room)	+++	+++	+++	++	++	++	++
	4°C	+++	+++	+++	+++	++	++	++
	-20°C	+++	+++	+++	+++	+++	+++	+++
	-80°C	+++	+++	+++	+++	+++	+++	+++

system) survived for several weeks both with and without cells. In the absence of any buffering system it is likely that these membranes became highly acidic due to the presence of CO_2 and H_2O. At room temperature (21°C in our laboratory) membranes started to take up water and become brittle after 3 months. At 4°C γ-irradiated membranes were stable for 6 months. However there was no visible or measurable change in fiber diameter at -20°C (Fig. 1) and -80°C (not shown) whether membranes were γ-irradiated or not. The inclusion of silica orange desiccant proved useful in seeing changes in water content in the environment which is from yellow to white.

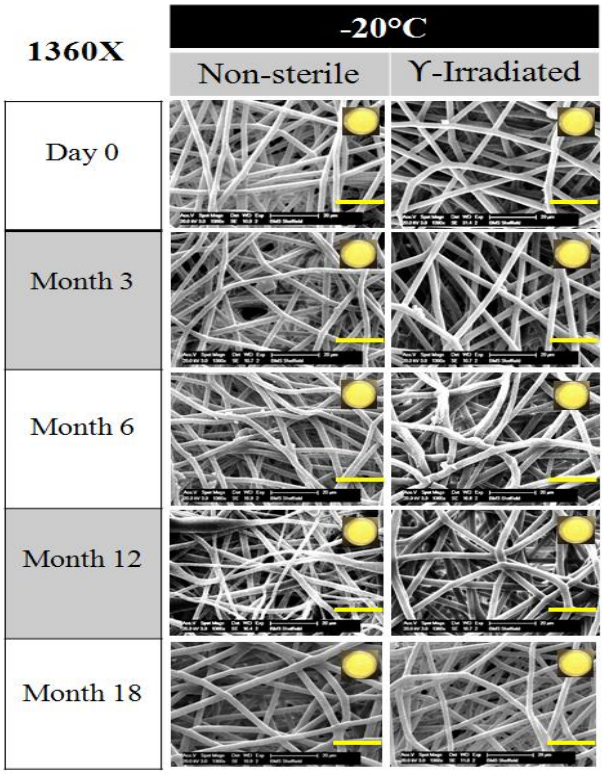

Figure 1. Comparison of fiber integrity of γ-irradiated and non-sterile electrospun PLGA (50/50) membranes (M_w:44 kg/mol) at -20°C during storage over periods of 18 months, Scale bar = 20µm. Changes in water absorption by membrane were detected using silica orange.

B. Storage of membranes – Vacuumed vs. non-vacuum

Storage of PLGA membranes at a similar range of temperatures for 12 months within vacuum packed bags showed that vacuum packing significantly improved the storage of the membranes (Fig. 2). At 37°C (under dry conditions) membranes showed some evidence of breakdown by 3 weeks. At UK and India room temperature membranes became more stable and started to take up water after 6 and 5 months respectively. At 4°C membranes were stable for 12 months which shows a great improvement compared to non-vacuumed condition. Membranes stored at -20°C and -80°C still gave optimum results (Fig. 3).

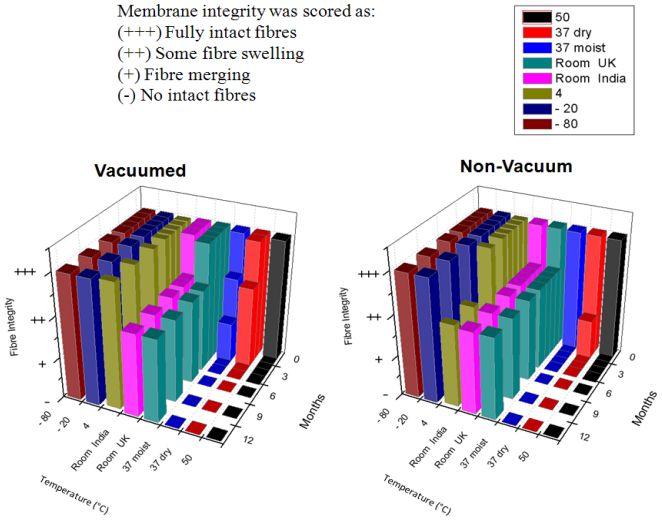

Figure 2. Effect of temperature and time on storage of vacuum and non-vacuum packed PLGA (50/50) membranes (44 kg/mol) over 12 months. Membrane integrity was scored as fully intact fibers (+++), some fiber swelling (++), fiber merging (+) or no intact fibers (-).

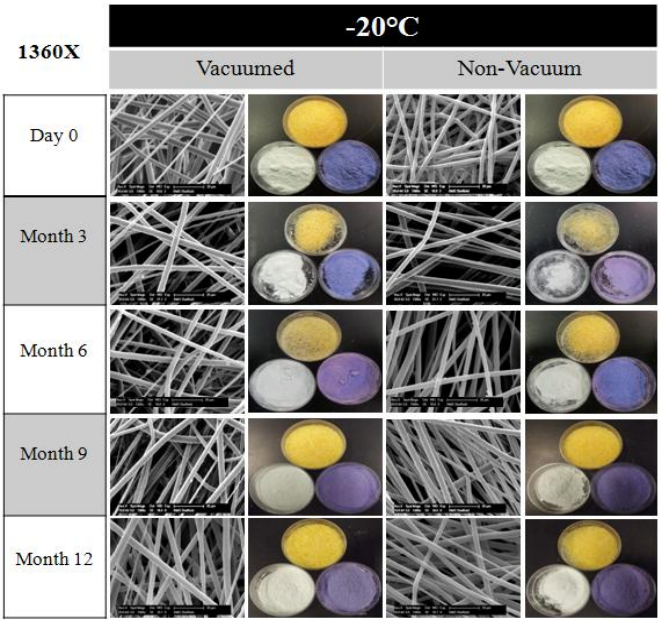

Figure 3. Comparison of fiber integrity of vacuumed and non-vacuumed electrospun PLGA (50/50) membranes (M_w:44 kg/mol) at -20°C during storage over periods of 12 months, Scale bar = 20µm. Changes in water absorption by membrane were detected using silica orange, cobalt (II) chloride and copper (II) sulphate desiccants.

C. *The use of a medical grade bag on the storage of membranes*

We explored the contribution of the use of a medical grade bag (PET/Foil/LDPE) to achieve a longer shelf life. Preliminary results show that this high moisture barrier bag significantly improved the storage of PLGA membranes even at higher temperature such as room temperature and 37°C dry. For instance, membranes stored at 37°C dry showed some evidence of breakdown by 6 weeks which is a great improvement in storage shelf life. This experiment is still on-going.

D. *Overall Storage results*

Overall results showed that vacuum packing significantly improved the storage of PLGA membranes. There was no visible or measurable change in fiber diameter for membranes stored at -20°C and -80°C. There was no visible colour change seen for -20°C and -80°C using silica orange, cobalt (II) chloride and copper (II) sulphate desiccants. Table 2 summarizes the impact of temperature on storage of PLGA membranes.

Table 2. The impact of temperature on storage of PLGA membrane.

Temperature	Integrity of fibers
-80°C	Intact up to 18 months (ongoing)
-20°C	Intact up to 18 months (ongoing)
4°C	Good to use for 12 months
Room Temperature (UK)	Good to use for 6 months
Room Temperature (India)	Good to use for 4 months
37°C	Degrade after 4 weeks
50°C	Degrade after 1 week

We have recently reported that both cultured cells and cells from limbal explants grow well on electrospun PLGA (44 kg/mol) with a 50:50 ratio of lactide and glycolide and sterilized with γ-irradiation which had been stored at -20°C for 12 months [14]. The results clearly showed cell outgrowth from explants onto these membranes after 2 weeks and that cells expressed the proliferation marker p63 suggesting that the membrane is able to support the growth of limbal epithelial cells even after 12 months in storage.

IV. Conclusion

In conclusion, sterilised PLGA membranes are suitable for further evaluation as a clinical alternative to the amniotic membrane in the treatment of limbal stem cell deficiency. There was no visible or measurable change in fiber diameter for membranes stored for 18 months at -20°C and -80°C whether membranes were γ-irradiated or not. Both vacuum packing and a high specification medical grade bag improved the storage of PLGA membranes. This synthetic sterilised carrier offers a lower disease risk alternative for clinical use than donor amniotic membrane with the capability of storage without loss of fiber integrity for up to 18 months at -20°C and 12 months at 4°C.

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